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(54) Method for determination of creatine kinase.

(57) In a method for determining the creatine kinase activity in e.g. serum, where the sample is brought in contact with ADP, creatine phosphate and a bioluminescence reagent having a stable light emission, AMP is added in a concentration which significantly inhibits the disturbing adenylate kinase activity without affecting the light emission.

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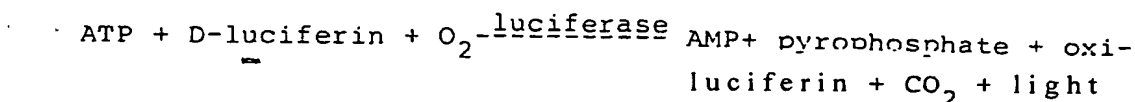
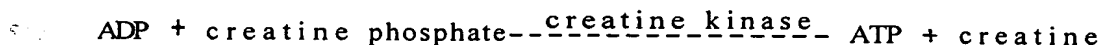
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Method for determination of creatine kinase.

The present invention refers to a method for determination of creatine kinase. More specifically, the invention refers to a technique in which the enzyme creatine kinase and its two substrates ADP and creatine phosphate are brought in contact with a bioluminescence reagent having a stable light emission and comprising firefly luciferase, D-luciferin and certain metal ions, such as magnesium ions, whereafter the light emission is measured. The bioluminescence reagents having a stable light emission proportional to the ATP concentration are described by Lundin and Myhrman in the Swedish Patent Application 7806296-5. The concentration of the ATP formed by the creatine kinase reaction can be continuously monitored by a bioluminescence reagent of this type and the creatine kinase activity can be measured as the rate of increase of the light emission. The reaction formulas are as follows:



The hitherto used method for the above determination has been elaborated by Lundin and Styrelius (Clin. Chim. Acta 87 (1978) 199). The bioluminescence reagent then used had analytical properties similar to those described in the Swedish Patent Application No. 7806296-5 but was in certain respects badly controlled and partly unspecified and was furthermore not possible to produce routinely. The method described by Lundin and Styrelius did however appear to be much more sensitive than the corresponding spectrophotometric method (see the comparison in the above reference by Lundin and Styrelius). The sensitivity was even adequate for determination of the subunit B of creatine kinase, which activity is more cardiac specific than a determination of the total creatine kinase activity, also in serum from healthy people. Results showing this have been published by Lundin, Lindberg,



Nordlander, Nyquist and Styrelius (Publication in press, presented at the International Symposium on Analytical Applications of Bioluminescence and Chemiluminescence, Brussels, September 1978). This is of big clinical
5 interest since it makes possible to discover also small myocardial infarctions during an early stage of the infarction process.

The method described by Lundin and Styrelius has however been less suited for clinical routine use depending partly on the
10 fact that the bioluminescence reagent used by Lundin and Styrelius has not been possible to produce routinely, partly because of the fact that ADP concentrations saturating the creatine kinase reaction have not been possible to use. The background of the first disadvantage is described in the
15 Swedish Patent Application No. 7806296-5. The reason why a saturating ADP concentration has not been possible to use has been that the forming of ATP through the adenylate kinase reaction, which disturbs the analysis, at an increase of the ADP concentration increases more than the creatine kinase
20 activity. The result is consequently that low creatine kinase activities are not possible to detect due to the adenylate kinase activity. This problem is described by Lundin and Styrelius and by Lundin and cooperators in the above references. The disadvantages of not using non-saturating ADP con-
25 centrations in the determination is firstly that the creatine kinase activity expressed as the formation of ATP per unit of time is lower than in the conventional spectrophotometric analysis which is confusing for the operator, secondly that the creatine kinase activity is dependent upon the ADP con-
30 centration, which means that small errors in this concentration directly results in an error of the determination of creatine kinase.

In the less sensitive spectrophotometric determination of creatine kinase, the adenylate kinase is inhibited by milli-
35 molar concentrations of AMP. Lundin and cooperators (reference see above) tried to use this method for inhibiting the adeny-

- late kinase in the bioluminescence method but found that the inhibition of the luciferase reaction was approximately just as big as the inhibition of the adenylate kinase (already a Concentration of 0.1 mM AMP gave rise to an inhibition of approximately 50%). The inhibition of the luciferase reaction was disturbing mainly due to the fact that it was dependent upon the concentration. Thus, a small error of the AMP concentration would change the luciferase activity and give rise to an error at the determination of the creatine kinase activity. Even if the addition of AMP made it possible to use a saturating concentration of ADP which in fact was never shown, the determination of creatine kinase would still be dependent upon the concentration of the AMP.
- According to the invention it has been shown that AMP also in rather high concentrations can be added without inhibiting the light emission of a bioluminescence reagent having a stable light emission provided that the determinations are performed at a pH value around or just below 7. This is shown in Fig. 1 which is a diagram showing the light emission as a function of the AMP concentration. In Fig. 1 the upper curve shows the situation at a pH value of 7.5, the next curve shows the situation at a pH value of 6.7 and the lowest curve shows the situation at a pH value of 6.5. The reaction mixture (1.0 ml) comprised 200 μ l bioluminescence reagent having a stable light emission and being produced in accordance with the Swedish Patent Application No. 7806296-5, 0.01 mol/l creatine phosphate and the above defined quantities of AMP. Thus, it can be seen that saturating concentrations of ADP can be used in the determination and in spite of this a very significant inhibition of the adenylate kinase reaction is obtained.

Even if the invention is not limited to any specific theory concerning the reaction mechanism enabling an addition of AMP in concentrations which significantly inhibit the adenylate kinase reaction without any significant effect on the light emission from the luciferase reaction detected, the most likely

reason is that this addition activates the light emission in the yellow green wavelength range simultaneously as the red emission is inhibited. Lee and McElroy (Arch. Biochem. Biophys. 145 (1971) 78) have described a similar, spectral displacement of the light-emission under conditions which do not give stable light emission. Since an important part of the light emission is constituted by red light under the conditions preselected at the determination of creatine kinase (pH 6.7) one should in order to decrease the inhibition of the light detected choose a light detector which has a high ratio for the sensitivity of yellow green and the sensitivity of red light. The higher AMP concentrations one wishes to add the higher this ratio has to be in order to compensate the inhibition of the red light emission by the activation of the yellow green emission. In very high AMP concentrations also the yellow green light emission is inhibited and such AMP concentrations can consequently not be added. Furthermore, it is according to Lee and McElroy (reference see above) possible that the activation of the yellow green light emission at an addition of AMP is due to the fact that luciferase changes its conformation. It is consequently important to choose such conditions for the analysis that this change of conformation can take place.

The advantages of determining creatine kinase in the presence of AMP according to invention are important as compared to techniques known per se. The addition of AMP implies that the adenylate kinase activity can be inhibited in order to make possible to determine the activity of the subunit B of creatine kinase in serum from healthy people. In addition to the determination of the creatine kinase activity independently of smaller variations of the ADP concentration the method according to the invention makes it possible to express the activity of the formation of ADP per time unit just as big as according to the spectrophotometric routine method as shown in Figure 3 which is a diagram showing the correlation between the spectrophotometric and the bioluminescence method for determining of creatine kinase in a serum sample. This is

important when the results are to be evaluated by the clinical chemist. A further advantage is that since the reaction rate of the creatine kinase reaction is higher, it is possible to use less serum for the determination.

5 This is important since it is often difficult to obtain sufficient amounts of serum and furthermore a certain interference on the luciferase reaction was obtained from serum at the concentration (50 μ l per ml reaction mixture) used by Lundin and Styrelius (reference see above). At low

10 concentrations of serum in the determination the luciferase activity is not affected by the addition of serum and it will not be necessary to determine this addition by adding a known amount of ATP for each sample. The analysis procedure is hereby considerably simplified. This consequence of the

15 procedure according to the invention and the fact that the reaction rate is higher will imply that the measuring time for the analysis is considerably reduced (less than 30 seconds for determining of the total creatine kinase activity). This is an important advantage both compared to the previous-

20 ly known bioluminescence determination of creatine kinase activity and the corresponding spectrophotometric method. This advantage is specifically important for instance when automatic analysers are used for the determination where the number of analyses performed per hour is important.

25 Thus, the technical advantages obtained according to the process of the invention in determining creatine kinase according to the bioluminescence method makes it possible to perform the analysis in clinical routine work and makes it possible to produce commercial kits for this purpose. The

30 clinical and economical significance of this is realised from the fact that worldwide several tenths of million determinations of the creatine kinase activity are carried out per year. The determination according to the invention has through its higher sensitivity and simplicity compared to the present

35 technique big possibilities to obtain a considerable part of this market.

The invention will now be illustrated with the following

non-limiting examples:

Example 1

This example, the results from which are shown in Fig. 2, shows that the inhibition of the adenylate kinase activity is higher in the presence of 1 mM AMP, and that the luciferase activity, is higher in the presence of 1 mM AMP and furthermore that the luciferase activity detected as an increase of the light signal when adding 10^{-7} ATP is independent of the additions of 0.5 mM ADP (Fig. 2a), 0.5 mM ADP + 1 mM AMP (Fig. 2b), 0.5 mM ADP + $2 \cdot 10^{-7}$ M diadenosin-pentaphosphate (Fig. 2c) and 0.5 mM ADP + 1 mM AMP + $2 \cdot 10^{-7}$ M diadenosin-pentaphosphate (Fig. 2d). The reaction mixture did contain in addition to the ADP reagent (ADP + possible additions as defined above) 0.2 ml ATP Monitoring Reagent (bioluminescence reagent with a stable light emission, LKB-Wallac, Article No. 1250-121, one vial dissolved in 10 ml distilled water) and 0.7 ml buffer, pH 6.7 comprising imidazolacetate, EDTA and N-acetylcystein so that the final concentration of 1 ml total volume was 0.1 M, 2 mM and 20 mM, respectively. After adding 20 μ l serum, ADP reagent was added so that the total volume obtained was 1 ml.

The adenylate kinase activity is calculated by dividing the increase of the light signal per minute by the stepwise increase of the light signal at the ADP addition whereafter the result is multiplied by the final concentration of the ATP added (0.1 μ l) and the dissolving factor for the serum sample ($1000 \mu\text{l}/20 \mu\text{l} = 50$). The adenylate kinase activity thus obtained is expressed in $\mu\text{mol}/\text{min}/\text{l}$ or U/l.

Example 2

This example shows the composition and use of a kit for determining creatine kinase in human serum. The number of determinations per kit is 50. The kit contains the following units (one of each):

ATP Monitoring Reagent (LKB-Wallac, article No. 1250-121)

ATP Standard (LKB-Wallac, article No. 1250-122)

ADP reagent (25 μ mol ADP, 50 μ mol AMP and 0.01 μ mol diadenosinpentaphosphate)

5 CP reagent (500 μ mol creatine phosphate)

CK buffer (45 ml buffer containing 0.11 M imidazol)

2.2 mM EDTA and 22 mM N-acetylcystein adjusted to pH 6.7 with acetic acid).

10 All these reagents except the buffer are present in freeze dried state. For determining of creatine kinase-MB the kit does also contain one bottle of anti-M antibodies from goat (Merck, Darmstadt) in freeze dried state.

Before use the ATP Monitoring Reagent is dissolved in a CK buffer (the mixture having a volume of 45 ml is called CK reagent below), ATP Standard is dissolved in 10 ml distilled water, ADP reagent in 1 ml distilled water, CP reagent in 1 ml distilled water. When determining the creatine kinase-MB also the anti-M antibodies are dissolved in the mixture or ATP Monitoring Reagent and CK buffer (CK reagent).

20 The determining of creatine kinase MB is performed according to the following procedure:

- 1) 20 μ l serum is added to 0.9 ml CK reagent containing anti M antibodies and is incubated at 25^o C for 15 minutes.
- 25 2) 20 μ l ADP reagent is added and the adenylate kinase activity is measured.
- 3) 20 μ l CP reagent is added and the sum of the adenylate kinase and creatine kinase activities are measured.

30 In each series of samples also a reagent blank without any addition of serum is added and furthermore a control serum having a known creatine kinase activity is analysed. Hereby, 10 μ l ATP Standard is added as an internal calibration in order to make it possible to transform the light intensity

value into an ATP concentration value according to the same principle as has been described in Example 1 for adenylate kinase determination.

- 5 When determining the total creatine kinase activity in serum it is normally unnecessary to measure the low adenylate kinase activity which remains in the presence of AMP. In this case ADP and CP reagents can be mixed whereby 40 μ l of the mixture is added (steps 2 and 3 according to the procedure above are made as one step).
- 10 This procedure facilitates the determination: but cannot be used when determining low activities of ~~cre~~ creatine kinase subunit B.

We claim:

1. Method for the determination of creatine kinase activity in for instance serum, whereby the sample to be determined is brought in contact with the two substrates of the creatine kinase reaction', ADP and creatine phosphate and a bioluminescence reagent having a stable light emission, based on firefly luciferase and D-luciferin, whereafter the rate of increase of the light emission is measured, which rate constitutes a measure of the creatine kinase activity, characterized in that AMP is added in a concentration significantly inhibiting the disturbing adenylate kinase activity without any significant affection of the light emission derived from the luciferase reaction.
2. Method according to claim 1, characterized in that AMP is added in a concentration of 10^{-4} - 10^{-2} M.
3. Method according to claim 1, characterized in that ADP is added in a concentration of 10^{-5} - 5×10^{-3} M.

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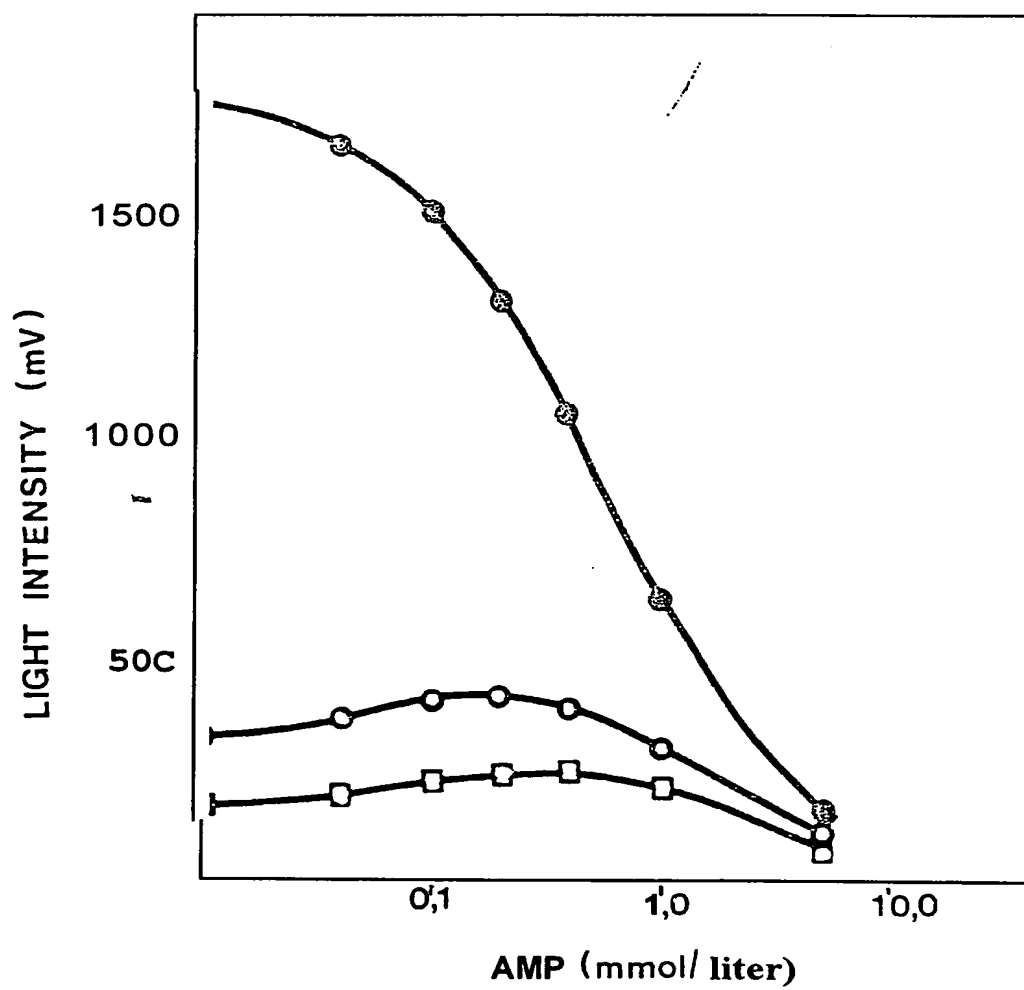


Fig.1

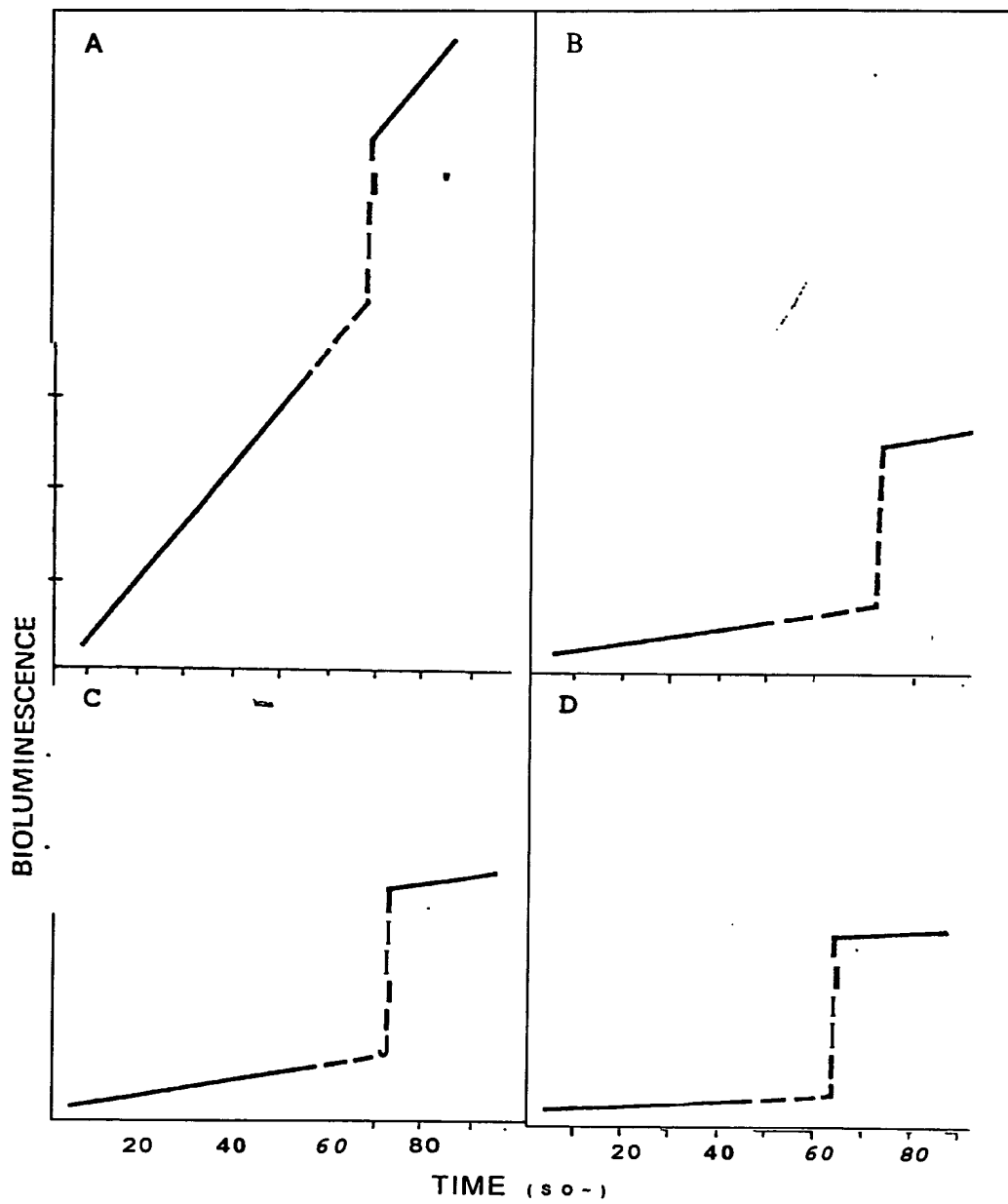


Fig. 2

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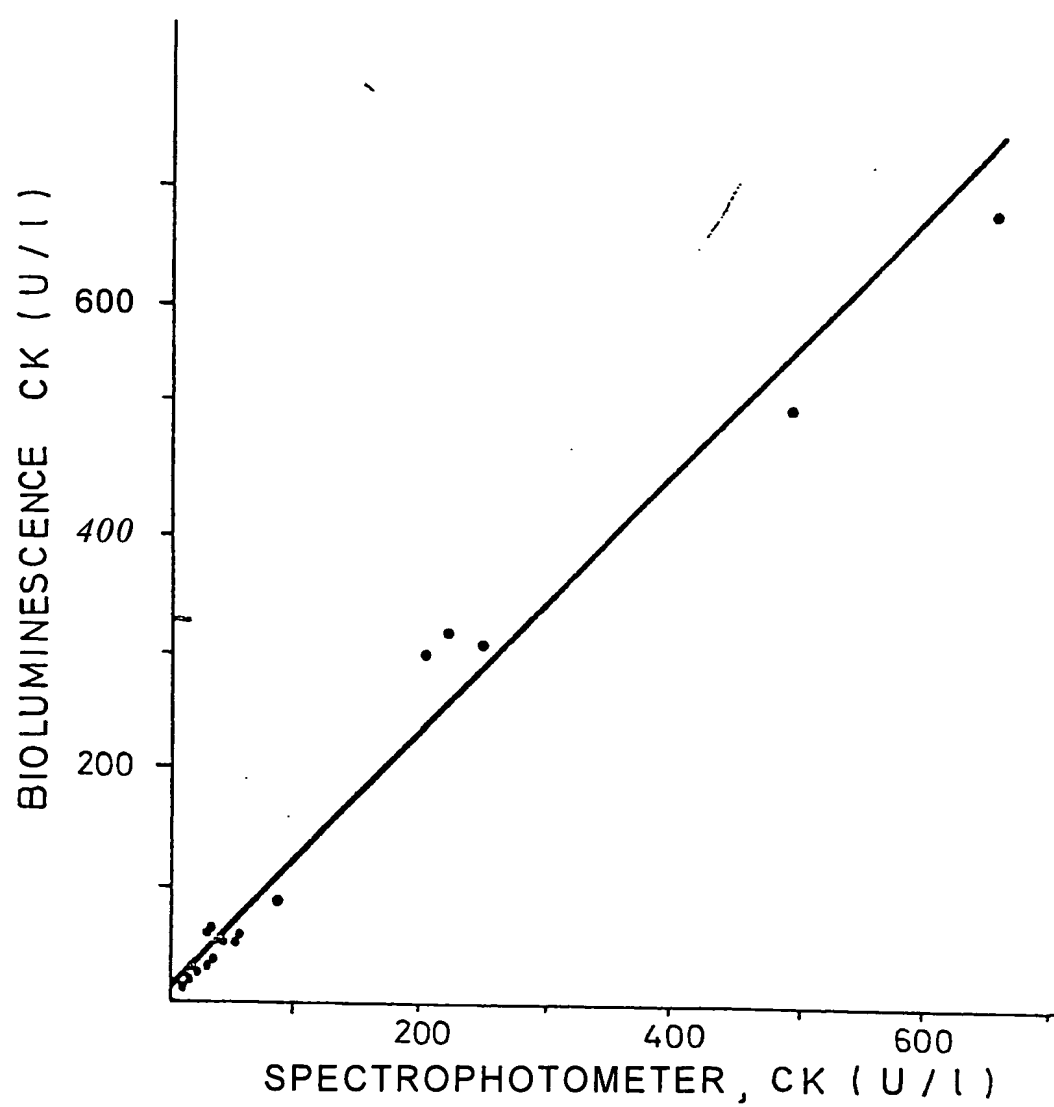


Fig. 3



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EUROPEAN SEARCH REPORT

0022432

EP 80 85 0093

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	CHEMICAL ABSTRACTS, vol. 92, no.5, February 4, 1980, abstract no. 36513h, page 314. Columbus, Ohio, USA DENTON MARK S. et al. "Continuously referenced, on line monitoring of creatine kinase and lactate dehydrogenase isoenzymes for use in clinical diagnostics", & Chromatogr. Sci. 19,9, 12 (Biol./Biomed. Appl. Liq. Chromatogr.2), 165-91. * Whole abstracts *	1	C 12 Q - 1/50 1/66
X	CLINICAL CHEMISTRY, vol. 12, no.5, 1972, pages 473-475. WILLIAM A. WARREN "Activation of serum creatine kinase by dithiothreitol". * Page 473 *	1,3	C 12 Q 1/50 1/66
X	CLINICA CHIMICA ACTA, vol 40, 1972, pages 133-138. HANNU SOMER et al. "Demonstration of serum creatine kinase isoenzymes by fluorescence technique" * Pages 134-136 *		CATEGORY OF CITED DOCUMENTS * Particularly relevant * technological background * non-written disclosure * intermediate document theory or principle underlying the invention conducting application document cited in the application citation for other reason
X	GB - A - 1 163 409 (CALEBIOCHEM) * Exemple *		member of the same patent family corresponding document
	GF - A - 2 005 830 (AMERICAN HOSPITAL SUPPLY CORP.) * Claims 7-9 *		
<input checked="" type="checkbox"/> The present search report has been drawn up for all claims			
The Hague		Date of completion of the search	Examiner





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DOCUMENTS CONSIDERED PO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl. 7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
	<p><u>US - A - 4 097 338</u> (KONTTINEN) * Example VII *</p> <p>--</p> <p>HANS ULRICH BERGMAYER: "Methoden der curymetischen Analyse", vol. 2, Verlag Chemie, 1974 Weinheim, DE STREHLER B.L. "Adenosin-5'-Triphosphat und Creatinphosphat. Bestimmung mit Luciferase", pages 2163-2177. * Pages 2172-2177 *</p> <p>--</p> <p><u>US - A - 4 001 088</u> (ALAN S. ANTONIK) * Abstract; column 2, lines 15-68; column 3, lines 1-20; claim 1 *</p> <p>--</p> <p><u>US - A - 4 080 265</u> (ALAN S. ANTONIK) * Abstract; column 2, lines 1-68; column 3, lines 1-10 *</p> <p>--</p> <p>D CHEMICAL ABSTRACTS, vol. 89 no. 17, October 23, 1978, page 229, Abstract 142374m, Columbus, Ohio, USA LUNDIN, ARNE et al. "Sensitive essay of creatine kinase isoenzymes in human serum using M Subunit inhibiting antibody and firefly luciferase".</p> <p>./.</p>	<p>1</p> <p>1-3</p> <p>1</p> <p>1</p> <p>1</p>	<p>TECHNICAL FIELDS SEARCHED (Int. Cl. 7)</p>



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DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl. ...)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
	& Clin. Chim. Acta, 1978, 87(2), 199-209. * Whole abstract *		
			TECHNICAL FIELDS SEARCHED (Int. Cl. ...)

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